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Effects of Pigment-Protein Fraction from *Nannocloropsis oculata* on TNFα and IL-6 which Act as an Anti-Inflammatory Against Viral Nervous Necrosis (VNN) Infection

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Abstract

The anti-inflammatory effect caused by VNN infection in the grouper fish using pigment-protein fraction of *N.oculata* has studied. Methods were to explore the pigment-protein fraction of *N.oculata*, in vivo test, and measurement of anti-inflammatory response using dot blott and immunohistochemistry technique. Results indicate that pigment-protein fraction of *N. oculata* was able to suppress grouper tissue inflammation when VNN infection was occurring. Inflammation sign was reduced by administration of pigment-protein fraction. This reaction was reinforced by raising of expression of TNFα and IL-6, which means that TNFα and IL-6 acts as an anti-inflammatory in fish tissue.

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Keywords: Anti-inflammation; IL-6; *N. oculata*, TNFα; VNN RNA

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1. Introduction

The grouper fish, especially *Cromilepte saltivelis* is an Indonesian export commodity to foreign fishin the Asian and European countries. Indonesia as one of the country culturing this fish commodity, requires the free pathogens commodities include viruses such as viral nervous necrosis (VNN). Infections of RNA virus, VNN, in grouper commodity are still a major problem for grouper culture. VNN is the cause of reduction of marine fish production in the worldwide. It also leads to mass mortality[1,2] till 100 % in larva stadia by the retinopathy and encephalopathy[3]. It is quite harmful and able to spread quickly[4].

Currently, many natural materials that are safe and environmentally friendly are used for the health management of grouper. One of the materials used for the development of anti VNN is a pigment-protein fractionfrom marine microalgae of *Nannochloropsis oculata (N. oculata). N. oculata* is a single cell marine micro algaethat called marine Chlorella. Several references have already mentioned that one of the inflammatory effects caused by exposure to ligands or viruses as pathogen to host cells causing an inflammatory reaction/inflammation. Inflammation is a response to the reaction of the binding of ligand with receptors on host cells because of the virus infection (viral RNA). The aim of this study is to observe the anti-inflammatory effect expressed by tumor necrosis factor alpha (TNFα) and Interleukin-6 (IL-6) caused by VNN infection in the grouper fish using treatment of pigment-protein fraction of sea microalgae of *N.oculata* to inhibit a transcription gene process of viral.

2. Material and methods

2.1. Place and time of research

The research was conducted in the Laboratory of Aquatic Sciences and Marine Biotechnology, Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia. It was carried out from February to June 2014.

2.2. Isolation of pigment-protein fraction from N. oculata.

Harvested cells of *N. oculata* (150 g wet weight) was homogenized with a mortar for 1 h after adding liquid nitrogen. Eight mL of 50 mM glycine and 20 mMKCl (pH 7.5) were added to the homogenate, then it was centrifuged at 12 000 g for 60 min at 4 °C. To the resulting supernatant, saturated ammonium sulphate solution (100 % sat.) was added in increments to a final concentration to 30 % saturation. This solution was centrifuged at 15 000 g for 30 min at 4 °C and recovered the supernatant. Dialysis tube was sterilized by boiling in a solution of 0.1mMTris-EDTA (pH 7.3) for 10 min before use. The sample was dialyzed against 2 000 mL of 20 mMTris-HCl, pH 8.0, for 24 h at 4 °C while stirring. After dialysis, the sample solution was filtered through amillipore filter (0.22 μm, Sartorius). These dialysis and filtration were repeated once. Protein contents of the solution after the second dialysis was measured spectrophotometrically at a wavelength of 280 nm using a nanodrop spectrophotometer (NanoDrop Technology, Wilmington, US). One absorbance is equivalent to 1 $\mu g \cdot mL^{-1}$ protein.

2.3. In vivo testing of pigment-protein fraction from N. oculata *on grouper*

The fish was acclimated in the batch (aquarium) for 7 d. Treatment was conducted by four groups of treatment, which are treatment A (control fish), treatment B (Fish + pigment-protein fraction), treatment C (Fish + pigment-protein fraction + VNN) and treatment D (Fish + VNN). Tests was conducted by oral (sonde method) using a hose feeding tube 6 times. Treatments were conducted in the first day, after 4 d, 8 d, 12 d, 16 d, and 20 d with volume of 307 mL, 321 mL, 331 mL, 336 mL, 346 mL, and 376 mL, respectively

2.4. Isolation of fish eye

After in vivo test had been finished, the fish was made slept using the clove oil and sea water. The fish was dissected to take out the eye and immersed it in the liquid nitrogen and stored in the liquid nitrogen tank.

2.5. Dot blot methods

Response of pigment-protein fraction from *N.oculata* as antiviral was confirmed by dot blott methods using Biorad's semi dry blotter device. The applied current was 300 mA for 30 min, followed by dyeing 2 % poncho containing 3 % trichloroacetic acid. After blot transfer to the nitrocellulose paper, it was rinseusing dH_2O to eliminate the poncho dye and then the paper was blocked using TBE at pH 7.4 with the supplement of 3 %.

2.6. Immunohistochemistry

Detection of both IL-6 and TNFα using immunohistochemistry was conducted according to Nanda et al.[5] Tissue with thickness of 6 μm was mounted on glass slides. Tissue of eye grouper was exposed using antimouse antibody then fixed in 2 % paraformaldehyde (pH 7.3), 10 min and then washed and incubated in 2 % blocking serum (from horse or goat), incubated overnight at 4 °C with primary antibody monoclonal anti-mouse IgG anti-IL-6 and anti-TNFα. After that, it was countered by secondary antibody anti-mouse IgG conjugated biotin for 30 min at ambient temperature. Avidin-biotin Complex peroxides kit (ABC-Elite, Merk Santa Cruz, Vector Laboratories) was used to detect the biotin using chromogen (diaminobenzidine tetrachloride). Tissue cut was dehydrated by an alcohol and cleaned with xylene.

2.7. Data analysis

Data was analyzed usingdescriptive analysis that is by comparing between control and after treatment. The quantification of the dot blot result was conducted using the software of ImageJ version 1.48.

3. Results

A Pigment-protein fraction from marine microalgae *N. oculata* was able to suppress a grouper tissue inflammation when VNN virus infection was occurring. The responses shown by TNFα and IL-6 were identified with anti mouse antibody labeling using TNFα and IL-6using Immunochemistry techniques. It is known that TNFα and IL-6 correlated to evoke the immune response in the fish cell in the process of antigen recognition pathways (signalling pathway) to the ligand or foreign object like VNN virus that infect grouper. The responses of inflammatory that cause infections of VNN are signs as like inflammation as swelling (cloudy swelling), the formation of channels in the tissue and also inflammation that result in bleeding (haemorrhage). The effect was reduced by administration of pigment-protein fraction of *N. oculata* into the fish. Treatment of pigment-protein fraction on fish as anti-inflammatory show reinforced by raising of expression of TNFα and IL-6, which means that the TNFα and IL-6 acts as an anti-inflammatory in fish tissue measured qualitatively by immunohistochemistry as shown in Fig. 1 and Fig.2.

Table 1.The response intensity of IL-6 and TNFα on eyes organ of grouper fish treated by pigment-protein fraction from sea microalga *N. oculata* through dot blot methods.

Item of dot blot	Control		Eye tissue response intensity (%)			
	K+	K−	Normal	+pigment-protein fraction	+VNN +pigment-protein fraction	+VNN
TNFα	100	0	81	45	36	96
IL–6	100	0	77	48	24	86

Table 1 shows that the change of immune response of grouper caused by in vivo test using a pigment-protein fraction of microalgae *N. oculata*. Eye organ in the grouper has epithelial cells. These cells are able to express a change in the immune response of TNFα and IL-6. The results of quantitatively response using dot blot test are

qualitatively confirmed by testing the response of TNFα by an immunohistochemistry technique in the eye organs as shown in Fig. 1 and Fig. 2.

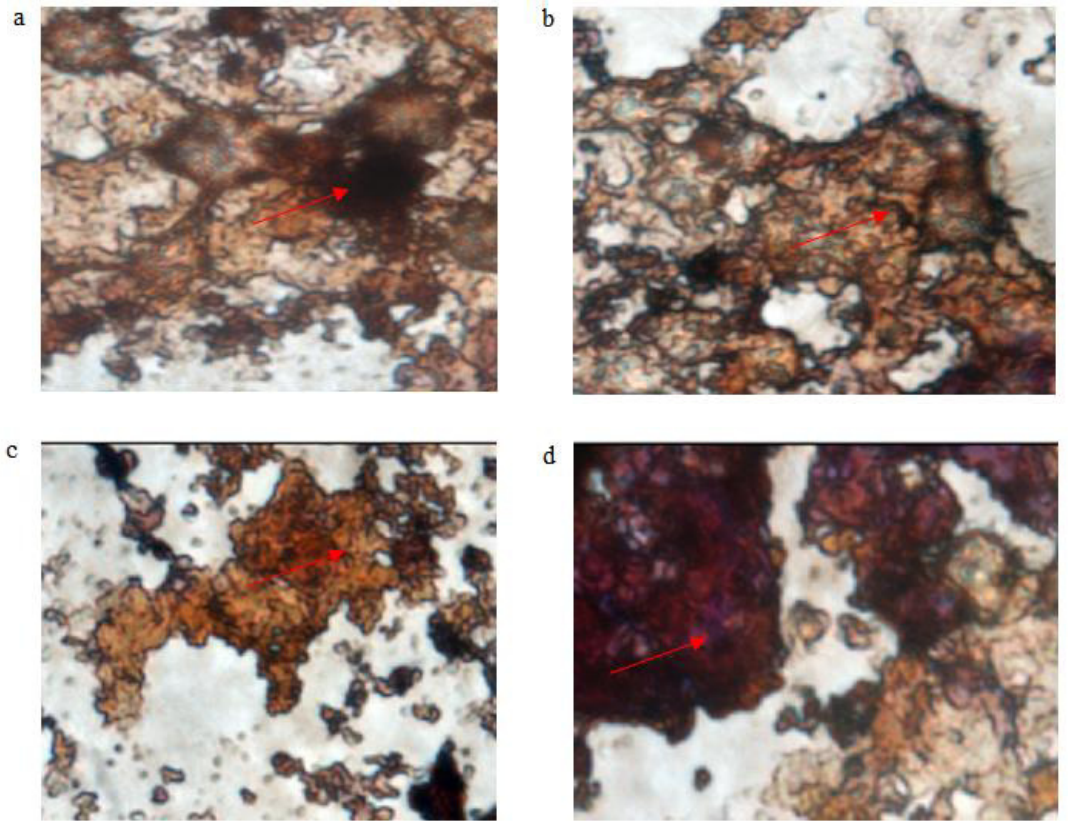

Fig. 1. Immunohistochemistry image to show the expression of TNFα in eye tissue of grouper after the following treatments: (a) normal eye (no treatment); (b) treatment by pigment-protein fraction; (c) treatment by pigment-protein fraction + VNN; (d) treatment by VNN

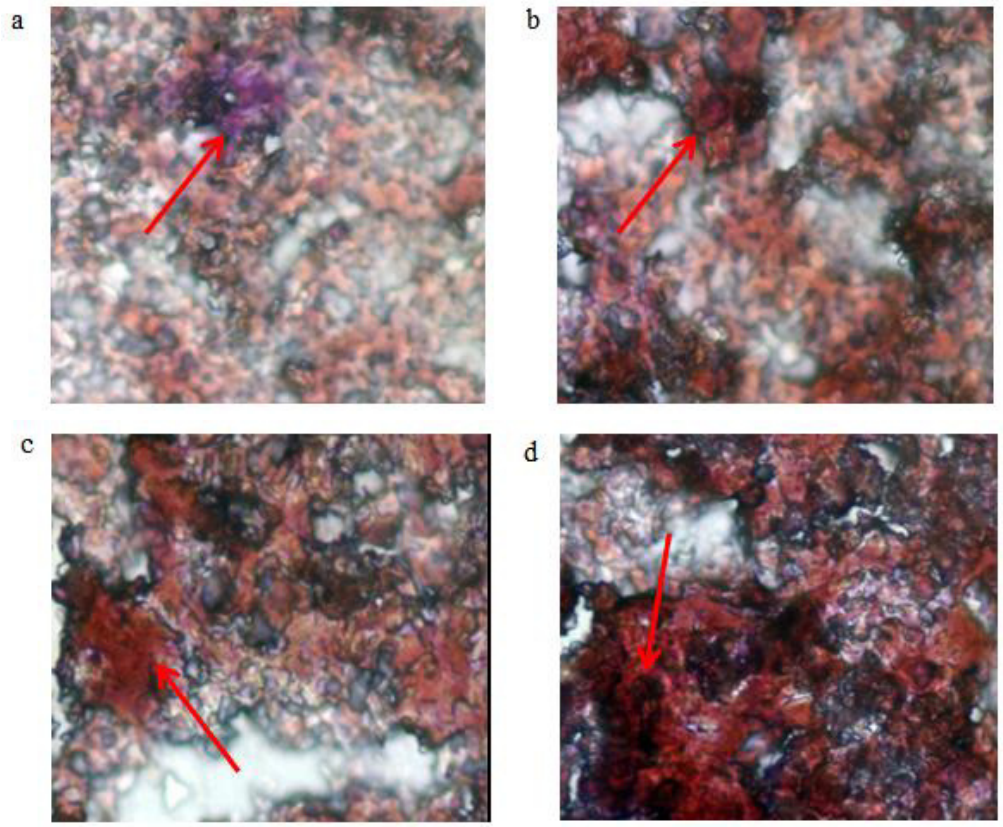

Fig. 2. Immunohistochemistry image to show the expression of IL-6 in eye tissue of grouper after the following treatments: (a) normal eye (no treatment); (b) treatment ofpigment-protein fraction; (c) treatment by pigment-protein fraction + VNN; (d) treatment by VNN

Fig. 1 and Fig. 2 show the qualitative response of TNFα and IL-6 in eye tissue, respectively. Response in the normal eye tissue (Fig. 1a and Fig. 2a) indicates the expression of TNFα and IL-6 labeled by secondary antibody anti-mouse IgG. TNFα and IL-6 has a stronger reaction indicated by the brown color using an SA-HRP substrate and reaction of TNFα and IL-6 appears to decline in the treatment using pigment-protein fraction of *N. oculata* (Fig. 1b and Fig. 2b), and increased response to infection of VNN, but the TNFα (Fig. 1a and Fig. 2a) response was decreased by reducing of the color response of the eye tissue grouper. The color reaction of anti-mouse antibody labelling of TNFα and IL-6 strongly increased in the treatment of VNN infection alone (Fig. 1d and Fig. 2d). In this treatment, TNFα and IL-6 response value exceeds even qualitatively eye organ responses without the treatment. This proves that there action of a viral infection causing an inflammatory reaction is adequately strong.

4. Discussion

VNN is a cause of disease of retinopathy and encephalopathy in grouper. Its eye is an attack target of RNA virus infection. Grouper eye is a good place for these RNA viruses to proliferate. It suggests that there is a match between the amino acids that exist in this VNN RNA virus and the receptor protein of grouper. Groupers have a simple body's immune system that is innate immunity and adaptive immunity[6]. One alternative RNA virus counter measures VNN is the provision of pigment-protein fraction, which is a protein[7] derived from marine microalgae *N. oculata*[8]. Pigment-protein fraction is a protein molecule which has functions to stimulate the release of viral proteins or RNA acts as a blocker for the VNN virus. Pigment-protein fraction in this treatment was administered

intramuscularly in the hope of striking directly on the blood vessels and can be deployed on the target RNA virus proliferation.

Pigment-protein fraction treatment of the physiological response of *N. oculata* marine microalgae as anti-inflammatory on VNN infected grouper was indicated by the expression of TNFα and IL-6 response. It proves that the pigment-protein fraction as a given protein in vivo has a function as an inducer of antiviral with the advent of TNFα and IL-6 expression. This response occurs in the body of fish because there is a reaction of immune cells that would activate an adapter molecule in the cytokine.

The expression of TNFα cells in the organs of fish eyes occurred with a pigment-protein fraction treatment and viral RNA was reduced. It means that the pigment-protein fraction from *N.oculata* acts as a reducing VNN virus infection. When it was compared with VNN treatment alone in organ grouper, the response seems to have a high viral infection, as indicated by the fact that the expression of TNFα value was higher than normal organ. Likewise the expression of IL-6 also gave the same pattern of immune response (IL-6) in the results of dot blot expression. This is reinforced by the opinions of Abbas and Lichtman[9] that the immune response against viral infections can be expressed through the function of immune cells include TNFα and IL-6. It is known that the grouper fish has immune systems that are adaptive or humoral. This immune response occurs after an administration of treatment for periods ranging between 4 d and 9 d post treatment of pigment-protein fraction *N.oculata*. Formed response is a good response to viral infection and also anti-viral. The quantitative response was reinforced by the qualitative examination using immunohistochemical techniques with secondary antibody anti-mouse TNFα. The reaction of VNN infection resulting an inflammation was strong enough in the eye tissue of grouper. Pigment-protein fraction from *N.oculata* treatment has functions to suppress viral infection up to half time to the normal response of the fish control. Adaptive immune response in grouper showed that the inflammatory reaction in the eye tissue of fish can be reduced by pigment-protein fraction. It was indicated that pigment-protein fraction acts as anti-inflammatory on VNN infection in grouper. It is known that TNFα and IL-6 are the kinds of protein that is released by cytokines. Cytokines are secreted endogenous proteins in cells, such as cells in the fish eye tissues.

Yarovinsky et al.[10] explains that this cytokine is a protein in the immune systems that regulate the interactions between cells and stimulate immune reactivity, both specific and non-specific. Responses to TNFα and IL-6 are an immune reaction to antigens such as viral infection RNA VNN. Pigment-protein fraction from *N. oculata* in this treatment was able to decrease the inflammatory response to infection of VNN.

5. Conclusion

This study was concluded that pigment-protein fraction from marine microalgae *N.oculata* is able to act as an anti-inflammatory antigen during an RNA virus infection in grouper fish.

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